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Maria Kitsara, Pierre Joanne, Solène Emmanuelle Boitard, Ibti hel Ben Dhiab, Barbara Poinard, et al.. Fabrication of cardiac patch by using electrospun collagen fibers. *Microelectronic Engineering*, 2015, 144, pp.46-50. 10.1016/j.mee.2015.02.034 . hal-01130222

HAL Id: hal-01130222

<https://hal.sorbonne-universite.fr/hal-01130222>

Submitted on 11 Mar 2015

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Fabrication of cardiac patch by using electrospun collagen fibers

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Abstract

Synergy between micro-nanotechnology and regenerative medicine can lead to new tools for health improvement. In this study, we investigate the efficacy of electrospun scaffolds - fabricated using clinically approved collagen – as supports for cardiomyoblast culture. The scaffolds were prepared using non-toxic solvents and crosslinking agents and characterized by scanning electron microscopy and contact angle measurements. Among different types of collagen samples, we found that atelocollagen can produce better quality of electrospun fibers than acid and basic fibrous collagen. Our results also show that the cell culture performance can be improved by adjusting the crosslinking conditions. Typically, increasing the concentration of citric acid of the cross-link agents from 5% to 10% w/w and the post-crosslink baking time from 1.5 to 2.5 h led to significant increases of the cellular colonization of the scaffold, showing three-dimensional growth of cardiac cells due to the specific morphology of the fibrous scaffolds. Finally, *in vivo* tests of the biocompatibility of the fabricated scaffolds have been done using a mouse model of dilated cardiomyopathy. As expected, the biocompatibility of the scaffold was found excellent and no visible inflammation was observed after the implantation up to two weeks. However, 5% citric acid electrospun collagen scaffolds was less resistant *in vivo*, proving again the importance of the processing parameter optimization of the electrospun scaffolds.

Highlights:

- Fabrication of collagen scaffold by electrospinning.
- Non-toxic collagen solvents and crosslinking agents.
- Biocompatible grafted collagen scaffolds in mice.
- Control of cardiomyoblast growth in collagen by crosslinking treatment.

Keywords:

electrospinning, collagen scaffold, biocompatibility, tissue engineering, cardiac cell therapy

1. Introduction

Engineered scaffolds have been widely used as structural and functional supports on which cells are seeded for the generation of cell therapy products. In the field of cardiac therapy, this approach is challenging but holds a real promise for improving function of the chronically failing myocardium [1]. Previously, a large number of investigations have been devoted to the culture of cardiac cells using scaffolds made of synthetic polymers. In particular, nanofibrous scaffolds obtained by electrospinning of poly-L-lactic acid (PLLA) and poly-(caprolactone) (PCL) could be produced for the formation of functional cardiac cell layers [2]. However, because of the lack of cell affinity these synthetic polymers are inherent less attractive than natural polymers - such as collagen, fibrinogen, elastin - for in vivo applications [3]. Among them, collagen constitutes one of the main proteins of the extracellular matrix, and this allows for a close simulation of the natural fibrillar structure of cardiac tissue [4]. Accordingly, it is interesting to consider electrospun collagen scaffolds as supports and carriers of cardiac cells that can be implanted for repair of the failing myocardium [5].

Different types of clinically approved collagen are now available but the ability of making fibrous scaffolds out of them has yet to be demonstrated. One of the issues is the crosslinking process, which uses chemical reagents to render the fibrous structures stable in the culture medium and after implantation. The most common crosslinkers for collagen fibers are glutaraldehyde - well known for its efficiency but somehow toxic - and combinations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) – a system which is less toxic but often causes a film-like morphology after the immersion of the fibers in water [6] [7]. Alternatively, a citric acid (CA) based system has been recently applied and shown the requested biocompatibility [8].

In this work, we study the electrospinning parameters of clinically approved collagen in order to produce optimal nanofiber scaffolds for three-dimensional culture of cardiac cells. In particular, we were interested in creating a patch form of scaffold that can be used for therapy purposes. Our results show that the colonization of cardiac cells is critically dependent on the crosslink parameters and that our collagen scaffolds are fully compatible with the implantation requirements, thereby providing a way towards a scaffold based cardiac cell therapy.

2. Materials and Methods

2.1 Collagen solutions preparation and electrospinning

Different types of clinically approved collagen, including atelocollagen, acid fibrous, basic fibrous were provided by Biom'Up (Saint-Priest, France) in form of dry material after lyophilization. For electrospinning, the raw collagen materials were dissolved using a solvent system containing ethanol, water and a variety of salts [7]. Specifically, the buffer solution is composed of the following salts dissolved in deionized water: potassium chloride, sodium chloride, potassium phosphate monobasic, sodium phosphate dibasic heptahydrate. The salts concentration is 20x in the collagen buffer solution. The final solvent system consists of buffer solution and ethanol in a ratio 1:1 v/v with a collagen concentration of 16 % w/v.

For the crosslinking of the collagen scaffolds a system containing CA, glycerol as extending agent and sodium hypophosphite (SHP) as catalyst was used [8]. The three crosslinking components were added to the collagen solution before the electrospinning. Different concentrations of CA and SHP were studied: 5% CA with 2.1% SHP and 10% CA with 4.2% SHP, keeping constant the glycerol amount to 3% (weight percentage based on the weight of collagen).

In electrospinning, the solution is fed through a thin needle opposite to a collecting plate and a high voltage is applied to form a jet of the solution that travels from the needle to the grounded collecting plate (fig. 1). In our study, the collagen solution was loaded to a syringe with a 23 gauge blunt needle and the flow rate was controlled via a pump at 1ml/h. By applying voltage of 13.5 kV and keeping a distance of 5cm between the needle tip and the grounded collector, fibers were deposited to the latter at room temperature (20 ± 2 °C). After 8 minutes of continuous electrospinning, collagen scaffolds were obtained that could be easily peeled off from the collector (fig. 2).

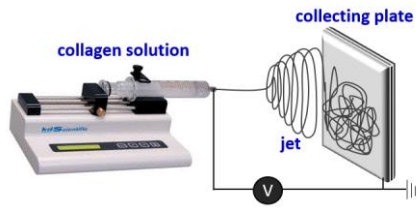


Figure 1. Schematic representation of the electrospinning apparatus.

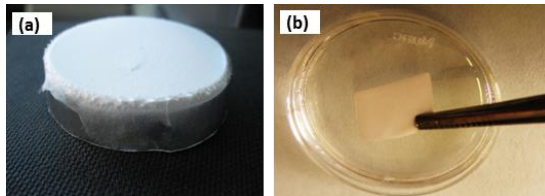


Figure 2. Images of the atelocollagen fibrous scaffold a) deposited onto aluminum collector, b) after the peeling-off.

2.2 Post-electrospinning treatment

After electrospinning, a crosslinking post-treatment was applied in order to make the scaffolds insoluble in cell culture medium and facilitate their handling. The carboxyl groups of CA reacted with the additional hydroxyl groups introduced by glycerol in order to form covalent bridges between the collagen fibers. The scaffolds were baked at 150 °C for three different periods (1.5h, 2h, 2.5h).

The crosslinked scaffolds were mounted onto handling devices for biologic assays. Cell crowns® holders were utilised for this purpose and then placed in 24-well plates for cell culture.

2.3 Characterization methods

The structure of the electrospun collagen scaffolds was studied after the steps of electrospinning and crosslinking treatment using scanning electron microscopy (SEM) and contact angle measurements.

The SEM measurements were performed using the e-LiNE (Raith). A thin gold layer of 5nm was deposited prior to measurements and images at different magnifications were obtained on at least three different areas in the same sample. The measurements were performed under extra high tension of 10kV.

The contact angle measuring instrument was the Drop Shape Analyzer - DSA 30 (Krüss). The sessile drop method was utilized and the measurements took place at room temperature (20 °C). The volume of the applied droplets of deionized water was 1.5 µl. The contact angle data were obtained by averaging over five measurements in different areas on the sample surface.

2.4 Cardiomyoblasts culture

The H9c2 cell line derived from embryonic rat heart tissue was used in this study. Cells were cultured in high glucose DMEM+Glutamax supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. They were seeded on the collagen scaffold after passage using trypsin-EDTA. After two days of culture, scaffolds were washed with PBS and fixed with 1% paraformaldehyde solution. After 2 washing steps, they were incubated with 5% bovine serum albumin (BSA) during 1 hour. Then Alexa Fluor® 488 Phalloidin (Life technologies) was used to stain the cells. After 3 washes in PBS, collagen scaffolds were mounted with mowiol. Images were captured using a motorized confocal laser scanning microscope (LSM 700, Zeiss).

2.5 Epicardial grafts

All procedures were approved by our institutional Ethics Committee and complied with the European legislation (Directive 2010/63/EU) on animal care. Conditionally-invalidated serum response factor (SRF) mice (MHC-MerCreMer:Sf/Sf) were used as recipients [9]. The Cre-mediated excision of floxed

SRF alleles in the heart was induced by daily intraperitoneal tamoxifen (28 $\mu\text{g/g/day}$; Sigma-Aldrich, France) injections on 3 consecutive days with the day of the first injection counted as day 1. Three weeks after tamoxifen administration, the animals underwent a left lateral thoracotomy after intraperitoneal ketamine (100 mg/kg; Merial, France)-xylazine (10 mg/kg; Bayer, Germany) anaesthesia and tracheal ventilation. Analgesia was performed for 2 days after surgery with a 10 mg/kg subcutaneous injection of ketoprofen (Merial). Acellular collagen scaffolds were sutured on the left ventricle. All animals were sacrificed at the end of experiment by cervical dislocation. Hearts were immediately removed, fixed in Tissue-Tek (Sakura, USA) and frozen in liquid nitrogen-cooled isopentane until they were sliced into 8 to 12- μm thick cryosections using a cryostat. The sections were stained with HE (hematoxylin and eosin) as described by Dubowitz [10]. Images were taken with an inverted microscope (Leica DMIL) equipped with a digital camera (Qicam, Qimaging).

3. Results and Discussion

3.1 Characterization of crosslinked scaffolds

3.1.1 SEM

Amongst the different collagen types, atelocollagen derived from native collagen by enzymatic extraction exhibited the best performance both in terms of dissolution to the benign solvent system (within few hours at room temperature) and of electrospinning ability. The acid fibrous collagen required one week to be dissolved and the basic fibrous collagen was not compatible with the used solvent system resulting to a partial dissolution after 3 weeks. All the types of collagen were tested for their ability to create fiber mats by electrospinning. Acid and basic fibrous types ended up to nanofibers with knots and it was not able to create a thick enough mat that could be unpeeled from the collector. These types of nanofibers might be used for the surface modification of cell culture substrates e.g. by directly depositing the electrospun fibers on the latter, but cannot be used as implantable scaffolds which is our intention. On the other hand, atelocollagen proved to be able to create uniform continuous fibers without knots (Fig. 3) that can be easily unpeeled from the collector (Fig. 2). Fig. 3a and 3b show the atelocollagen mesh-structured scaffold and demonstrate, at a higher magnification, the uniformity of the fibers with a diameter of $1\pm0.3\mu\text{m}$.

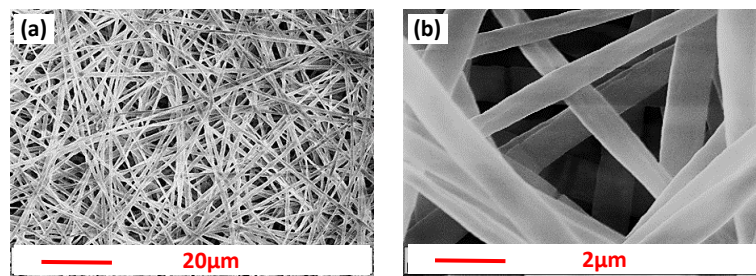


Figure 3. SEM images of atelocollagen fibers after electrospinning at different magnifications: (a) 1x and (b) 10x.

After the post-electrospinning crosslinking treatment, the samples were characterized before and after their immersion into deionized water in order to verify the efficacy of the method. Neither the duration of baking nor the concentration of the cross-linkers did affect the fibers morphology before the immersion to water.

Conversely, the morphology of the samples after the immersion in water varied in relation with the concentrations of CA and SHP. At low concentrations, loss of porosity was observed as shown in fig.4a, whereas the morphology was significantly improved for 10% CA and 4.2% SHP (fig. 4b,c).

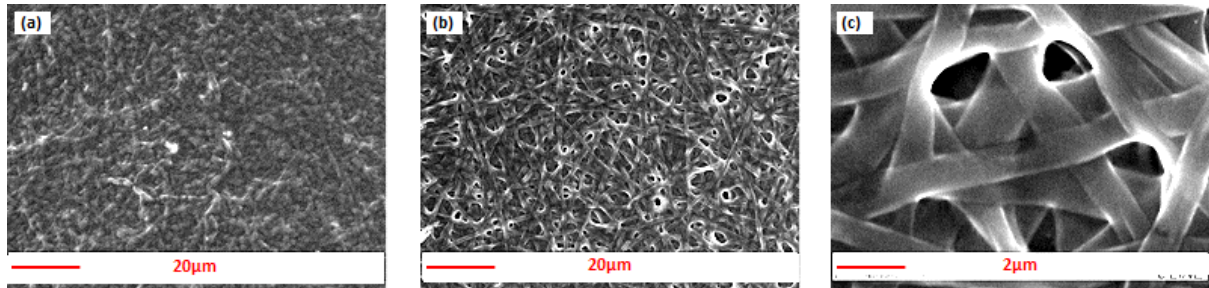


Figure 4. SEM images of crosslinked collagen scaffolds after immersion in water (a) 5% CA, (b) 10% CA and (c) 10% CA at higher magnification.

3.1.2 Contact angle measurements

Collagen scaffolds before and after the crosslinking treatment were then characterized. Figure 5a shows the surface of a non-crosslinked scaffold, five seconds after the deposition of water droplet, which spread immediately all over the scaffold, causing scaffold dissolution. For the measurements of the crosslinked scaffolds with 10% CA, the latter were immersed in water and then placed in cell crowns® holders. The water contact angles obtained with dry samples showed that the surfaces did not dissolve, but are hydrophilic with a contact angle of 50° just five seconds after the droplet deposition (fig. 5b). The water contact angles decreased down to 30° just 60 seconds after the droplet deposition, which further indicates the porosity of the samples (fig. 5c).

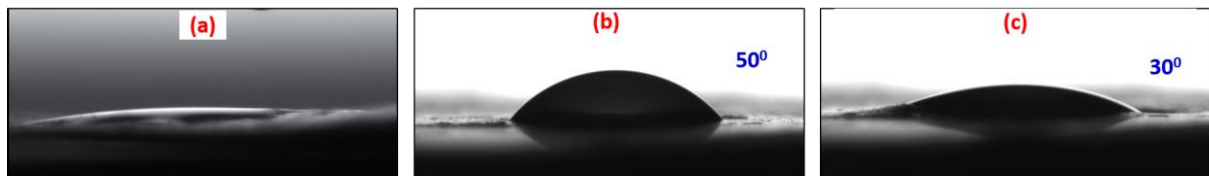


Figure 5. Images of water droplets on collagen scaffolds with 10% CA (a) uncrosslinked, (b,c) crosslinked captured at different time after droplet deposition.

3.2 Biological validation of the scaffolds

3.2.1 In Vitro Studies: Cellularisation of collagen scaffolds

The electrospun collagen scaffolds were successfully used for culture of H9c2 cells. Figure 6 shows a confocal microscopic image after immunostaining for actin and nuclei which demonstrates the colonization of the 10 % CA scaffold. Clearly, the cells were spread all over the scaffold and show a nice structure of their actin filaments (the blue cells are more deeply sunk into the collagen than the others). The cells seem to spread more extensively as the baking time was longer. It should be noted that, by increasing the baking time, the stiffness should be enhanced, which could be more desirable in our case. Moreover, comparing the different concentrations of CA, it is obvious that the higher baking time (2.5h) led to a greater extent of cell colonization.

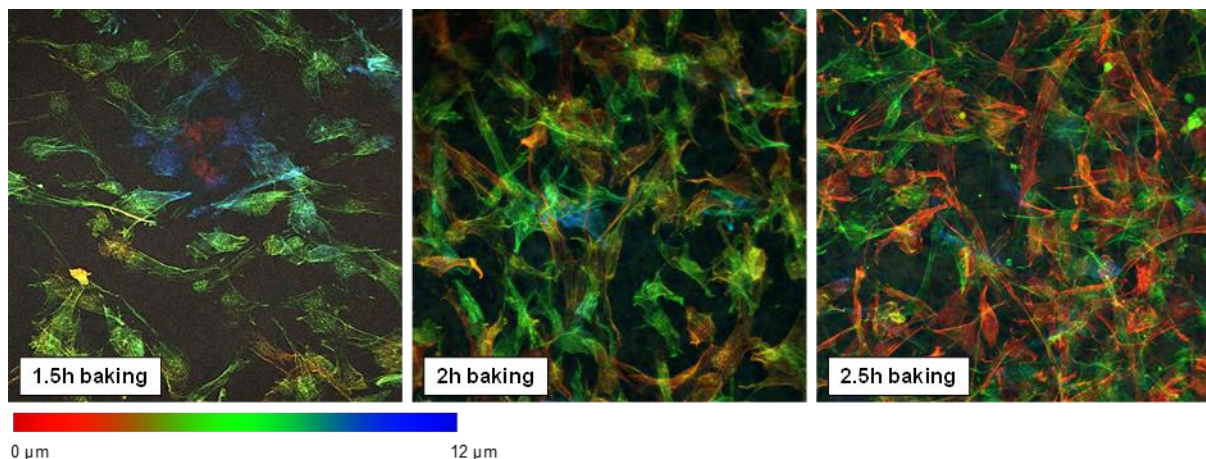


Figure 6. Confocal images of H9c2 cardiomyoblasts on the electrospun collagen scaffold (10% CA) after 48h cell culture.

Preliminary experiments with human pluripotent stem cell-derived cardiac progenitors have also been performed, showing a promising efficacy of the fabricated collagen scaffold. The next step will be the implantation of the cell-seeded scaffold in mice with failing myocardium with the hope that the scaffold may provide an optimal microenvironment to the cells, thereby allowing them to improve heart function, either by integrating structurally within the recipient tissue or, more likely, by activating endogenous reparative pathways.

3.2.2 In Vivo Studies: implantation of acellular collagen scaffolds

In this study, we used a double transgenic mouse model which allows the inactivation of SRF only in cardiomyocytes as tamoxifen-inducible Cre recombinase expression is under the control of the cardiomyocyte-specific alpha myosin heavy chain promoter [9]. Five days after tamoxifen injection there is a drop of SRF mRNA and protein levels. Then, mice exhibit a progressive dilated cardiomyopathy, leading to a non-ischaemic heart failure and death within 10 weeks. This model allows a timed control of the functional deterioration, thereby allowing a better comparison between treated and non-treated mutant animals.

Non-ischaemic heart failure can only be treated by drugs in contrast to ischaemic cardiomyopathies. If the treatment fails, the only remaining options are heart transplantation or permanent implantation of assist devices [11]. Our goal is to develop cell therapy as a new tool for the cure of non-ischaemic cardiomyopathies. The first issue to address is the biocompatibility of the nano-fabricated collagen scaffold. To this end, 10 conditionally-invalidated serum response factor (SRF) mice (aMHC-MerCreMer:Sf/Sf) were transplanted with the 10% CA collagen scaffold. The biocompatibility was found excellent, as the scaffold was detectable from 2 days (4 mice) to 2 weeks (6 mice) after the implantation, with no visible inflammation (fig. 7, A & B). It was slightly degraded over time as its thickness only decreased to 60% after two weeks of implantation (fig. 7, C). This decreased thickness also indicates that the inflammation is very low. Moreover, the porosity of the electrospun collagen scaffold did not significantly change between 2 and 14 days, indicating that the structure of the patch is not altered (fig. 7, D). It should be noted, that we also implanted 5 % CA electrospun collagen scaffolds in 12 mice. The results were very similar to the 10 % CA after 2 days of implantation. But after 2 weeks, we were not able to find the scaffolds in 62.5% of the implanted mice. This suggests that the 5% CA electrospun collagen scaffolds seem to be less resistant *in vivo*. Together, all these results demonstrate the good biocompatibility of the electrospun clinically-approved collagen scaffold in the context of dilated cardiomyopathy.

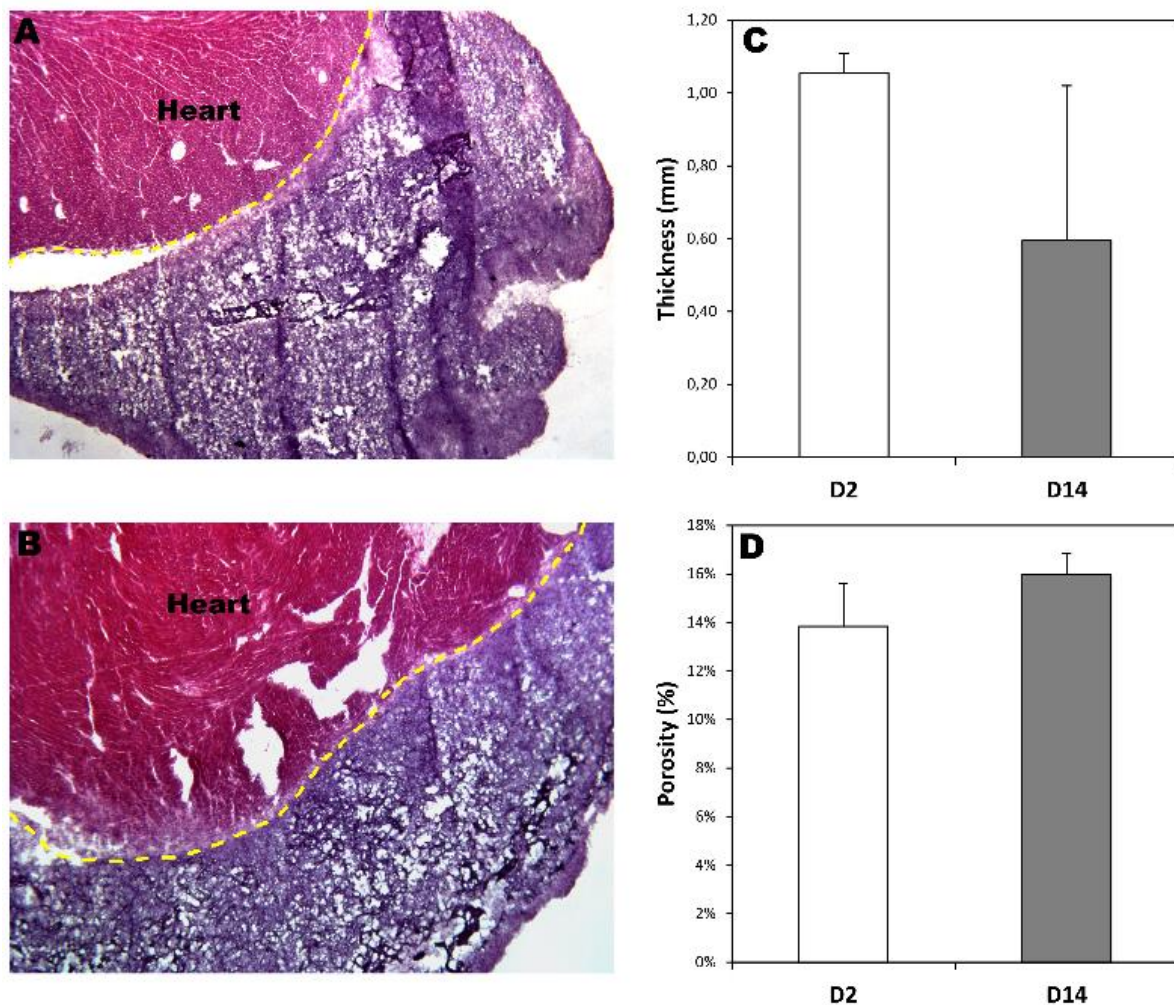


Figure 7. Biocompatibility of the electrospun collagen scaffold. Hearts of aMHC-MerCreMer:Sf/Sf mice were explanted 2 days (A) or 14 days (B) after implantation. The mean thickness (C) and the porosity (D) of the grafted patches were analyzed.

4. Conclusions

The present work has been designed to create a cardiac patch which can be used as a cell carrier for therapy purposes. Among different approaches, electrospinning appears to be the simplest but yet versatile method for the fabrication of fibrous scaffolds from a large variety of materials. Then, the challenge is to find the most suited material and processing parameters. Although the electrospinning techniques have been well documented, few of them applied to cardiac cell therapy which requires a full control of the porous size, the mechanical stiffness and the bio-reactivity or bio-degradability of the scaffold. As a first step of our approach, we produced nanofibers by electrospinning of a clinically proven collagen dissolved in a non-toxic solvent system in contrary to the widely used 1,1,1,3,3,3 hexafluoro-2-propanol [3]. In addition, we chose a citric acid-based system for cross-linking of the electrospun nanofibers, which makes our fabrication process compatible to the therapy purposes. After crosslinking, the collagen fibrous scaffold could be easily mounted on a standard device for in vitro studies. This should allow more systematic investigations to elucidate the dependence of cell migration and three-dimensional colonization as a function of pore size and thickness of the scaffold. The fabricated nanofiber scaffolds could also be used for in-vivo testing. Our preliminary results have shown the feasibility of implantation of this patch. Finally, the mechanical stiffness of the scaffold will have to be optimized in order to match that of the heart to be repaired.

Acknowledgements

This work was supported by the French National Research Agency (ANR-12-RPIB-0015) and by the Fondation de l'Avenir (ET2-658). P.J. was supported by a fellowship from the Fondation Lefoulon-Delalande and S.B. from the Conseil Régional d'Île de France (DIM Biothérapies). M.K. would like to thank Ms. Anne-Laure Vayssade from Microfluidique, MEMs and Nanostructures Laboratory of ESPCI for allowing her using their contact angle measurement instrument.

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